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For understanding the possible role of PCBs in breast cancer induction we have to 1. Unravel the mechanisms of PCB carcinogenesis, 2. Investigate whether these mechanisms occur in the breast. We found that: *i.* 4-chlorobiphenyl is an **initiator** in rat liver; *ii.* Human breast tissue is qualitatively able to metabolically activate PCBs similarly as the liver; iii. in mouse liver PCBs are activated to metabolites that bind to nuclear protein and DNA; the structure of the adduct still needs to be determined; iv. PCB quinone and hydroquinone metabolites bind to DNA *in vitro,* deplete intracellular glutathione and produce reactive oxygen species (ROS) in cells in culture; v. PCB treatment results in the activation of transcription factors, 8-OH-dG formation, and increase in lipid peroxidation derived endogenous polar DNA adducts in rat liver; vi. PCBs produce oxidative stress by redox reactions of the metabolites or/and by changes in the levels of anti-oxidant enzymes and cofactors; vii. Human breast tissue varies greatly in pro-/anti-oxidant enzyme activity. Oxidative stress could be the major factor in PCB carcinogenesis. Therefore we will analyze oxidative stress in breast tissue after PCB treatment, and try to identify enzymatic risk factors for PCB carcinogenesis in women.

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INTRODUCTION

Our research is aimed at the investigation of the possible involvement of polychlorinated biphenyls (PCBs) in breast cancer induction. PCBs are industrial chemicals which persist in our environment. The lipophilicity of PCBs and their tendency to bioaccumulate in adipose tissue and breast milk raise concern about the health risks associated with exposure to PCBs and related compounds. Commercial PCB mixtures are complete carcinogens, producing hepatocellular carcinomas in rats and mice, but the mechanisms by which they do so have not been determined. We and others have shown that higher halogenated PCBs (especially, tetra-, penta-, and hexa-chlorinated biphenyls) act as promoters of carcinogenesis, but the question remains whether PCBs have also DNA damaging and cancer initiating activity. In our original proposal we presented considerable data to support the concept that the lower halogenated biphenyls may be activated by hepatic and breast (milk) enzymes to oxygenated species that are electrophilic and bind to DNA. Of particular interest were the hydroquinone/quinone metabolites. Our data showed that PCBs are metabolized *in vitro* to dihydroxy metabolites and that these can be further oxidized by peroxidases (including lactoperoxidase) and prostaglandin synthase to quinones. We also presented data that these PCB-quinones are strong electrophiles that react *in vitro* with both sulfur and nitrogen nucleophiles, including nucleotides and DNA. We offered preliminary observations that supported the concept that PCB quinones may redox cycle with the production of reactive oxygen species that could pose an indirect risk to cellular DNA.

To build on these observations, we proposed the following: *(i)* determine if PCBs that accumulate in breast tissue are converted to dihydroxy metabolites and whether these metabolites can be oxidized by breast tissue subcellular fractions and lactoperoxidase to PCB quinones, *(ii)* characterize the DNA-adducts of breast-specific PCBs with regard to the specific metabolites formed and nucleotides involved and identify the chemical structure of the adducts, *(iii)* determine the biological consequences of DNA-adduct formation by PCB metabolites, including detection of single- and double-strand breaks, analysis of sites that block *in vitro* DNA synthesis and analysis of mutations, and *(iv)* employ *in vivo* models to identify PCB adducts and mutations in the breast, and investigate the possibility of using DNA adduction for human biomonitoring purposes by detecting DNA-reacting metabolites in serum and breast milk. These studies address our working hypothesis that PCB congeners that accumulate in the breast may be metabolized in this tissue to electrophiles, especially quinones, which then react with critical cellular targets, including DNA, and that these reactions lead to mutagenic events resulting in neoplastic change. Our project therefore addresses the question of the possible mechanisms of PCB carcinogenicity, with emphasis on the human breast as target organ.

STATEMENT OF THE WORK (from the original grant)

Synthesis of PCBs and PCB metabolites (service project, not included in specific aims)
This work has continued during the whole funding period as needed.

Aim 1: Metabolism studies with 2,4,4'-trichlorobiphenyl (see pages 7-9, and 14 ffl.)
Study 1: Analyze PCB metabolism by breast tissue and cells
Study 2: Investigate quinone formation from PCB metabolites by breast enzymes
Study 3: Study the production of oxygen radicals by PCB metabolism in breast tissue

Aim 2: Examination of adduct formation by PCB metabolites (see pages 10-13 and 21)
Study 1: Examine DNA-adduct formation from synthesized metabolites of 2,4,4'-trichlorobiphenyl
Study 2: Examine DNA-adduct formation from various PCBs during metabolism with breast tissue
Study 3: Isolate DNA-adducts of 2,4,4'-trichlorobiphenyl and characterize their chemical structure (work in progress)

Aim 3: In vitro mutagenicity studies with 2,4,4'-trichlorobiphenyl and metabolites
Study 1: Detection of strand breaks (see Ludewig et al., 1998)
Study 2: Analysis of site-specific blocks of DNA synthesis by adducts (work in progress)
Study 3: Examination of mutation induction by adducts in shuttle vectors in vitro (work in progress)

Aim 4: In vivo studies
Study 1: Detection of DNA-adducts and 8-OH-dG in breasts of rats (see page 21 and work in progress)
Study 2: Examination of gene mutations in breast tissue of transgenic rats (work in progress)
Study 3: Identification of PCB metabolites by analysis of DNA-reacting metabolites in serum and milk for biomonitoring and risk assessment (work in progress)

BODY OF THE REPORT

Our goal is to increase our knowledge of *i.* the metabolic activity of breast tissue and *ii.* the reactivity/toxicity of the PCB metabolites that are formed, so that we *iii.* learn to understand the mechanisms that are involved in PCB carcinogenicity in general and in the breast in specific. This knowledge is needed to make data-derived judgements about the potential risks of PCB exposure and to make informed decisions about protective and preventative actions.

Metabolism of lower halogenated biphenyls in liver and breast tissue.

Our first aim was to examine whether lower halogenated biphenyls are metabolized to reactive compounds in that tissue, where they preferably accumulate, the breast. This question is important, since the general conception is that metabolic activation occurs in the liver, implying that only the liver is a target organ for PCB carcinogenicity. Another question is whether the same metabolites are formed in liver and breast, if any are formed at all. In a previous report we have shown that lower halogenated biphenyls are metabolized by rat liver microsomes to various mono- and dihydroxylated compounds and that indeed the same metabolites can be found after incubation of, for example, 4-chlorobiphenyl (4-CBP, PCB 3) with microsomes derived from human breast tissue. We identified 3 monohydroxy- (2'-,3'-, and 4'-OH-4-CBP) and 3 dihydroxy- (2',3'-, 3',4'-, and 2',5'-diOH-4-CBP) metabolites. The differences in metabolism between human breast and rat liver were only quantitative, not qualitative. This suggests that the same metabolic activation mechanisms that occur in the rat liver may also occur in the human breast. The next question was whether PCBs can be found in the nuclei of organs *in vivo* and whether we can show that they were metabolically activated to reactive and therefore potentially harmful compounds.

PCBs bind to Nuclear Protein and to Nuclear DNA *in vivo*.

PCBs as persistent organic pollutants are considered to be fairly inactive and may not bind covalently to macromolecules. Our hypothesis is that PCBs, especially the lower halogenated biphenyls, are indeed metabolized to electrophilic species that react with critical cellular targets, including DNA. To test this hypothesis we conducted a specific study to determine if PCB congeners, like 4-CBP and 3,3',4,4'-tetrachlorobiphenyl (3,3',4,4'-CBP, PCB 77), can covalently bind to DNA and chromatin proteins *in vivo*. This would not only prove that the compounds, unmodified or the metabolites, reach the nucleus, but also, whether they could act as direct, initiating compounds by DNA adduction. The protocol for this assay, suggested by Lutz (1979) and further modified by Sagelsdorff et al. (1983), was used to determine the binding indices of these compounds to DNA and chromatin protein in female C57/BL6 mice (6/group). Benzo[a]pyrene (B[a]P) was used as a positive control and a negative control group was treated with corn oil only (vehicle). All chemicals were administered by intraperitoneal injections. The mice were pretreated with ß-naphthoflavone (100 µmol/kg) and phenobarbital (400 µmol/kg) for three consecutive days to induce hepatic enzymes prior to the administration of ^{14}C-labeled PCBs and B[a]P (1 mCi/kg). Animals were euthanized 24 hours later by anesthesia (Metophane) and exsanguination through cardiac puncture. DNA and chromatin proteins were extracted from whole livers (Sagelsdorff et al., 1983). The DNA extraction procedure consisted of a standard phenol-

chloroform extraction followed by further purification of the DNA by hydroxylapatite chromatography and dialysis. The DNA concentration was assessed spectrophotometrically and confirmed by postlabeling. The purity of the DNA was verified using a highly-sensitive protein assay (BCA). Chromatin proteins were precipitated from the phenol phase with acetone, and resuspended in SDS-phosphate buffer. The final protein concentration was assessed using the BCA assay. The radioactivity bound to the DNA and protein fractions was measured by scintillation counting and compared to the dose of chemical injected. The DNA or carcinogen binding index (CBI) and the protein binding index (PBI) were computed according to Sagelsdorff et al. (1983).

Our results show that both B[a]P and PCBs bind to nuclear targets, the DNA fraction as well as to chromatin proteins. Higher counts were found in the protein fractions than in DNA. The DNA samples contained less than 5 µg/ml of proteins, which supports the assumption that the radioactivity was associated with nucleic acids and not related to protein contamination of the samples. The number of adducts/nucleotide, the µmol chemical bound/amino acid and the means and standard errors for the computed CBIs and PBIs are listed in the table below. All three compounds bound strongly to proteins. The total binding was highest for 4-CBP, followed by B[a]P and 3,3',4,4'-CBP. The PBI of 4-CBP was about 3/5 of the B[a]P value, due to the higher amount of compound needed to achieve the same effect. 3,3',4,4'-CBP was the least active. The PBIs for all treatment groups were significantly different from the control group (95% confidence intervals comparison). All compounds also bound to DNA. The number of adducts per 10^9 nucleotides was similar for all 3 compounds, but about twice the dose of PCB was needed in comparison to B[a]P. The CBIs of the B[a]P-treated mice was significantly different from the corn oil-treated mice. CBIs for the PCB-treated groups showed a high variability (see dot-diagram below). Thus despite higher mean CBI values, the PCB-treated groups did not show a statistical difference with the control group when 95% confidence intervals were compared. These data indicate that *i.* PCBs do reach the nuclei of mice livers, *ii.* PCBs are activated *in vivo* to metabolites that bind to nuclear protein and DNA. This strongly suggests that PCBs could be genotoxic and therefore could be initiators of carcinogenesis. This hypotheses, that PCBs are inducers of carcinogenesis, still needed to be proven. Our next experiments were designed to address this question.

Table: Binding and binding indexes of B[a]P, 4-CBP, and 3,3',4,4'-CBP in mouse liver after treatment *in vivo*

Group	Dose	Adducts/10^9 Nucleotides	CBI	µmol bound / mole Amino Acids	PBI
Corn oil	0.4 ml i.p.	0	0.19 ± 0.1	0	$6.9 \times 10^1 \pm 25$
B[a]P	38 µmol/kg	6.7	1.81 ± 0.6 *	91.3	$2.4 \times 10^3 \pm 283$ *
4-CBP	83 µmol/kg	7.8	0.95 ± 0.4	122.05	$1.5 \times 10^3 \pm 246$ *
343'4'CBP	80 µmol/kg	6.1	0.77 ± 0.5	54.39	$6.8 \times 10^2 \pm 73$ *

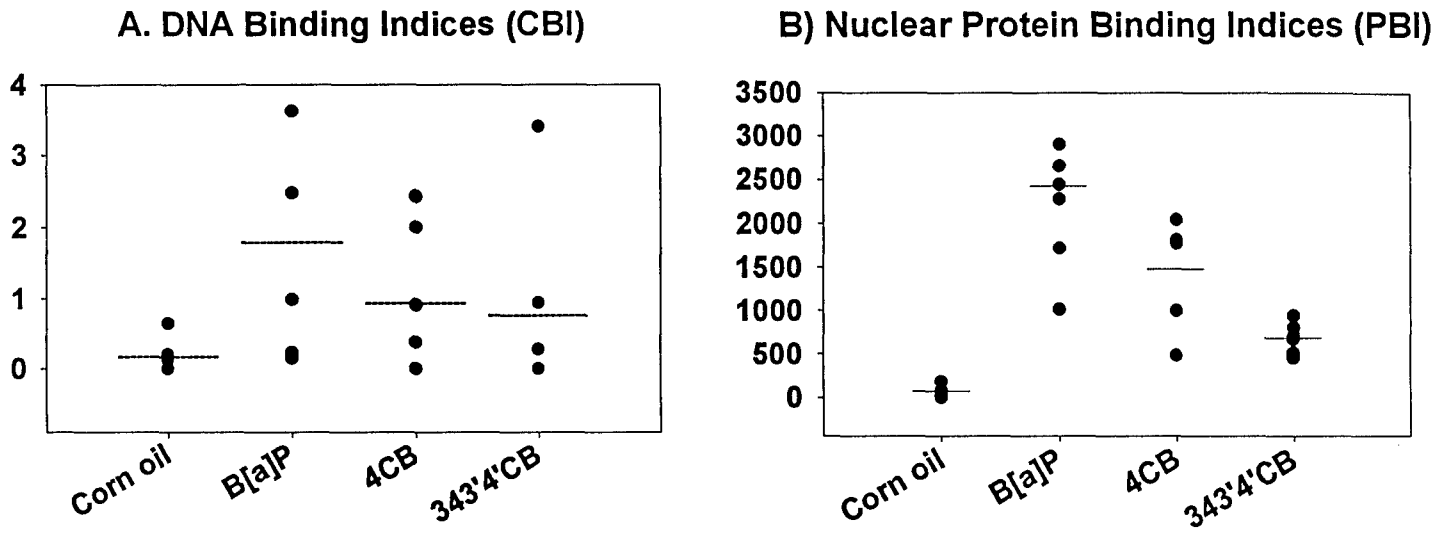
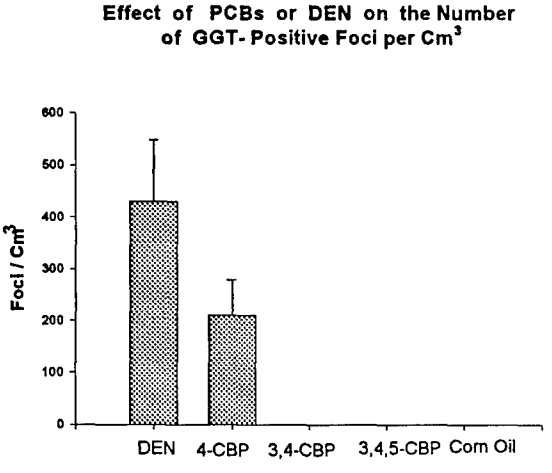


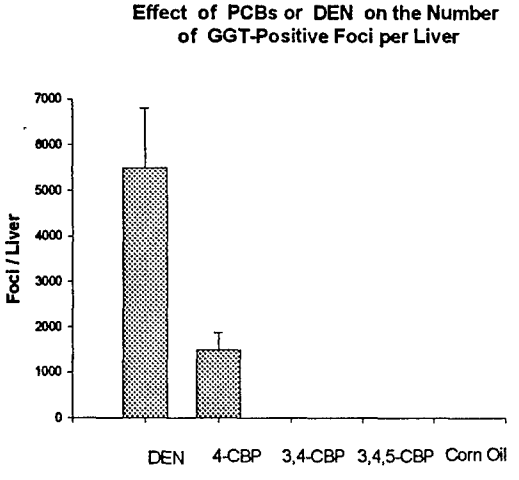
Figure. Dot diagram of CBI (A) and PBI (B) showing the data distribution in each group (each dot is one animal) as well as the mean (line). The value 0 represent values under the limit of detection of the scintillation counter.

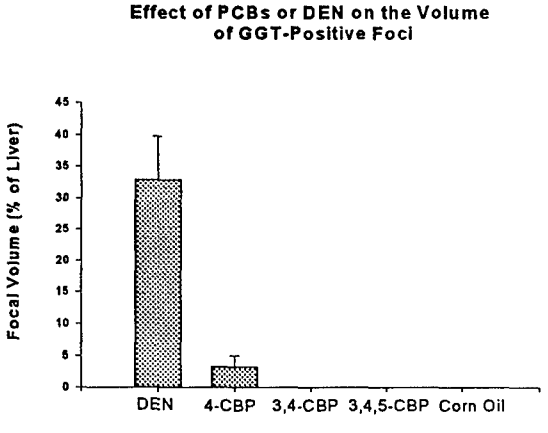
Solt-Farber Studies: 4-CBP does Initiate!

To determine if PCBs can act as initiators in an *in vivo* rat liver cancer initiation model, we have begun to test PCBs using a modified Solt-Farber protocol (Solt et al., 1977; Tsuda et al., 1980). We have now completed the testing of 3 PCBs. Male F344 rats were first subjected to partial hepatectomy, followed by a single dose of the positive control diethylnitrosamine (DEN, 100 mg/kg = 980 μmol/kg), or the test agent (600 μmol/kg), or vehicle (corn oil). Two weeks post-dosing the initiated cells were growth-selected by treating the animals with daily p.o. doses of 30 mg/kg 2-AAF (for 3 days), followed by a single p.o. dose of CCl_4 (2 mls/kg) and then 3 additional daily treatments with 2-AAF. Negative controls for each treatment group without the selection agents were conducted in parallel. One test agent representing a monochloro-, a dichloro-, and a trichlorobiphenyl was chosen as an initiator. Four weeks after treatment with the PCBs the animals were euthanized and the livers removed, weighed, and analyzed. No animals receiving corn oil, DEN, or PCB without selection had nodules. No nodules were also seen in animals receiving corn oil or 3,4-dichlorobiphenyl (3,4-CBP, PCB 12), or 3,4,5-trichlorobiphenyl (3,4,5-CBP, PCB 38), as initiators plus selection. All animals receiving DEN + selection showed visible nodules. Also, the mono-chloro congener, 4-CBP, induced grossly visible nodules in 50% of the treated rats. Histological staining of liver sections further revealed the presence of foci and nodules in hematoxylin- and eosin-stained sections in all DEN + selection treated animals and in 80% of 4-CBP + selection treated animals. The figures below show the number of gamma-glutamyltransferase (GGT)-positive foci (expressed per cm^3 and per liver) and total focal volume (in % of liver volume). DEN-treated animals had 425 foci/cm^3, 5500 foci/liver, and 34% of the total liver converted to foci, while 4-CBP treated animals had 200 foci/cm^3, 1500 GGT-positive foci/liver, occupying 3% of the total liver. The other PCBs did not significantly increase the number or volume of GGT-positive foci. No GGT-positive foci were detected in vehicle or non-selected groups. The number of

foci per liver is especially high with DEN, since these animals showed an increase in total liver weight. Also, the foci in the 4-CBP+selection group were relatively small, compared to the DEN+selection group. However, 4-CBP and DEN have very similar potency, when the number of foci/cm^3 per µmole compound is compared (0.33 and 0.43, respectively). This proves to our knowledge for the first time that a PCB, in this case 4-CBP, can act as an **initiator** of liver carcinogenesis. We have preliminary data with other congeners also inducing foci formation. These data have not been statistically analyzed as yet and therefore were not included in this report. The next step was to analyze the possible mechanisms. Our hypothesis is that PCBs initiate carcinogenesis either indirectly, by generating reactive oxygen species (ROS) or directly, by binding to the DNA. The most accepted mode of cancer initiation is through a direct interaction of the compound with the cellular DNA or DNA adduct formation by an activated metabolite. Thus we further investigated DNA adduction by PCB metabolites *in vitro* and PCBs *in vivo*.







Formation of DNA adducts with PCB quinones and hydroquinones *in vitro*

Our previous studies have indicated that mono- di- and trichloro biphenyls can be biotransformed to DNA-reacting species when incubated with microsomes, followed by incubation with peroxidases, including lactoperoxidase (reviewed in Robertson & Gupta, 1999). This suggested that it was a quinone metabolite that reacted with the DNA. Since

then considerable efforts have been made in the preparation of sufficiently pure quinone derivatives to test this hypothesis. Since the *ortho-* (2',3'- and 3',4'-) quinones are less stable we focussed our studies on *para-* (2',5'-) quinones.

Calf thymus DNA (300 μg) was incubated with *para-q*uinone derivatives of PCB 3, PCB 12 and PCB 38 (4-, 3,4-, and 3,4,5-CBP, respectively) in the presence of 50 mM Tris.HCl, pH 7.4. Following incubation at 37°C for 4 h, the purified DNA was analyzed by nuclease P1-mediated ^{32}P-postlabeling assay. All three quinones tested showed two major and two to four minor DNA adducts (Fig. below). Quantitation of adducts showed that at equimolar concentrations, PCB 38 was most reactive (298 adducts/10^9 nucleotides), followed by PCB 3 (140 adducts/10^9 nucleotides). PCB 12 was least reactive (56 adducts/10^9 nucleotides). The extent of DNA adduction, however, varied significantly at different pH values, with pH 9.5 resulting in the highest adduction (1220 and 740 adducts/10^9 nucleotides for PCB 3 and PCB 12, respectively; PCB 38 could not be tested at this pH due to insufficient quantity). This confirms our hypothesis that PCB-quinones can react with DNA to form adducts. The migration pattern of these adducts of synthesized quinones with DNA can now be used as standards to identify quinone-DNA adducts formed *in vivo*.

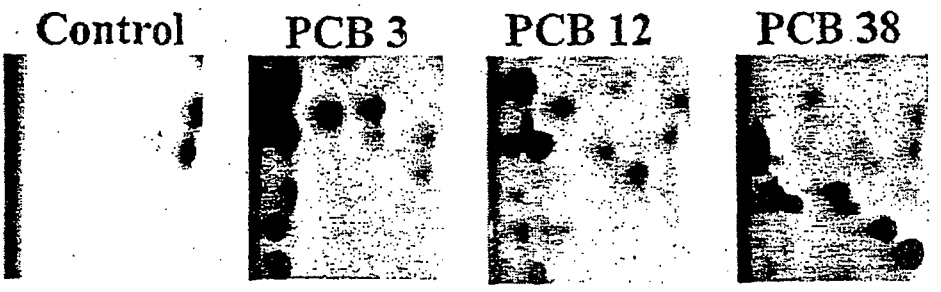


Figure: ^{32}P-Adduct maps of calf thymus DNA reacted with *para*-quinone metabolites of PCB 3, PCB 12, and PCB 38.

Further, the *para*-dihydroxy metabolites (hydroquinones) were also reacted with DNA and analyzed by ^{32}P-postlabeling. Several adducts of these PCB hydroquinones with calf thymus DNA were visible and guanine was found to be the most frequently involved DNA base. Also, 4-chlorophenyl-hydroquinone was about 4-8 times more active in DNA adduct formation then the 4-chlorophenyl-quinone. Efforts are under way to identify the individual adducts formed with the hydroquinone (or semiquinone ?) and the quinone.

Detection of direct DNA adduction *in vivo*

In order to further strengthen our hypothesis that PCB-quinones could directly be involved in PCB carcinogenesis, we attempted to find quinone-DNA adducts in animals that were treated with PCBs. Female Sprague-Dawley rats (6-10 rats/group) were pre-treated i.p. with corn oil or a mixture of phenobarbital (PB) & β-naphthoflavone (β-NF) or higher chlorinated PCBs (PCBs 77 and 153) for three consecutive days. PB+β-NF and higher chlorinated PCB are known to induce metabolizing enzymes, which should increase our chances to find adducts. Immediately after pre-treatment the animals

received for 3 days i.p. injections of PCB 12, or PCB 38 (100 μmol/kg body weight). The rats were euthanized 24 h post-treatment and liver, kidney, and breast tissues were collected. Analysis of the isolated liver DNA by nuclease P1-version of the ^{32}P-postlabeling assay showed one DNA adduct in PCB 38-treated animals as compared to vehicle treatment. This extra spot was present in both pre-treatment groups (PB+β-NF or PCB, data not shown) and is therefore not related to the compounds used for enzyme induction, but a consequence of PCB 38 treatment. So far, however, we were not able to identify the chemical nature of this spot. Comparison of the *in vivo* adducts showed no chromatographic resemblance with any of the adducts formed by interaction of the synthesized *para*-quinone of PCB 38 with DNA *in vitro*. No "extra" adduct spot was detected in the PCB 12-treated liver DNA. This shows that PCB-DNA adducts *in vivo*, if formed at all, are extremely difficult to detect or may even be below the detection limit of our assay at this time point. These data seem discouraging, and the breast tissue was therefore temporarily saved and stored at –70° C. It is premature to rule out the presence of direct adduction of PCB metabolites with DNA *in vivo*, but the method may need to be further optimized for their detection. New experiments in which rats have been treated with these PCBs for a longer period of time are underway to determine if adducts related to direct DNA adduction of PCB metabolites are formed *in vivo* or not. Interestingly, however, treatment with PCBs had a profound effect on the level of endogenous liver DNA adducts, a phenomenon presumably associated with ROS (see below).

Evidence of Indirect DNA damage: polar endogenous DNA adducts

As we discussed in the proposal and below, we believe that ROS may be involved in PCB carcinogenesis. ROS can react with cellular lipids, an event that is called lipid peroxidation, and thereby generate crotonaldehyde, malondialdehyde (MDA) and 4-hydroxynonnenal (4-HNE). These products are not only indicators of oxidative stress in the cells, but also highly reactive themselves. With support from an NCI grant (CA-77114), Dr. Gupta's laboratory has detected novel endogenous DNA adducts in rat liver at relatively high levels (2000 – 5000 adducts/10^9 nucleotides). Unlike previously known lipophilic endogenous DNA adducts (the so-called I-compounds) (reviewed in Randerath *et al.*, 1999; Gupta *et al.*, 1999), these new adducts are polar and migrate in the region of the chromatogram where cyclic adducts migrate. It is thus hypothesized that these polar endogenous adducts could originate from lipid peroxidation products. As a natural extension of our hypothesis and based on our previous observation that MDA is produced in rats treated with PCBs (Dogra et al., 1988), we were obviously interested to determine, if PCB treatment had any association with this new class of endogenous adducts.

Female Sprague-Dawley rats (6 rats/group) were treated i.p. with PCB 3, PCB 28, PCB 38, PCB 77, or PCB 153 (4-, 2,4,4'-, 3,4,5-, 3,3',4,4'-, or 2,2',4,4',5,5'-CBP, respectively) in corn oil, twice a week for 3 weeks. A group of rats received vehicle only. Animals were sacrificed 3 days after the last dose, and the liver DNAs were analyzed using nuclease P1-mediated ^{32}P-postlabeling assay. Another sample lacking DNA was also included as a negative control. All samples, with the exception of the negative control, showed qualitatively similar pattern of these adducts (Figure below, left side), but quantitatively three of the five PCBs (PCB 38, PCB 77 and PCB 153) showed a significant increases in the levels of these endogenous polar DNA adducts. The highest increase in these polar endogenous adducts was seen with PCB 77 (5-fold enhancement;

Figure below, right side), followed by PCB 153 and PCB 38. The observation that these endogenous DNA adducts migrate in the region of lipid peroxidation-derived DNA adducts (Figure, left side) suggest that they may originate from ROS, and PCB treatment presumably accelerate their formation. Although the identity of these polar endogenous DNA adducts is not clear yet, it nevertheless points to oxidative reactions induced by PCB treatment and this causes us to broaden our research efforts to include these polar endogenous adducts in our future analysis of breast tissue.

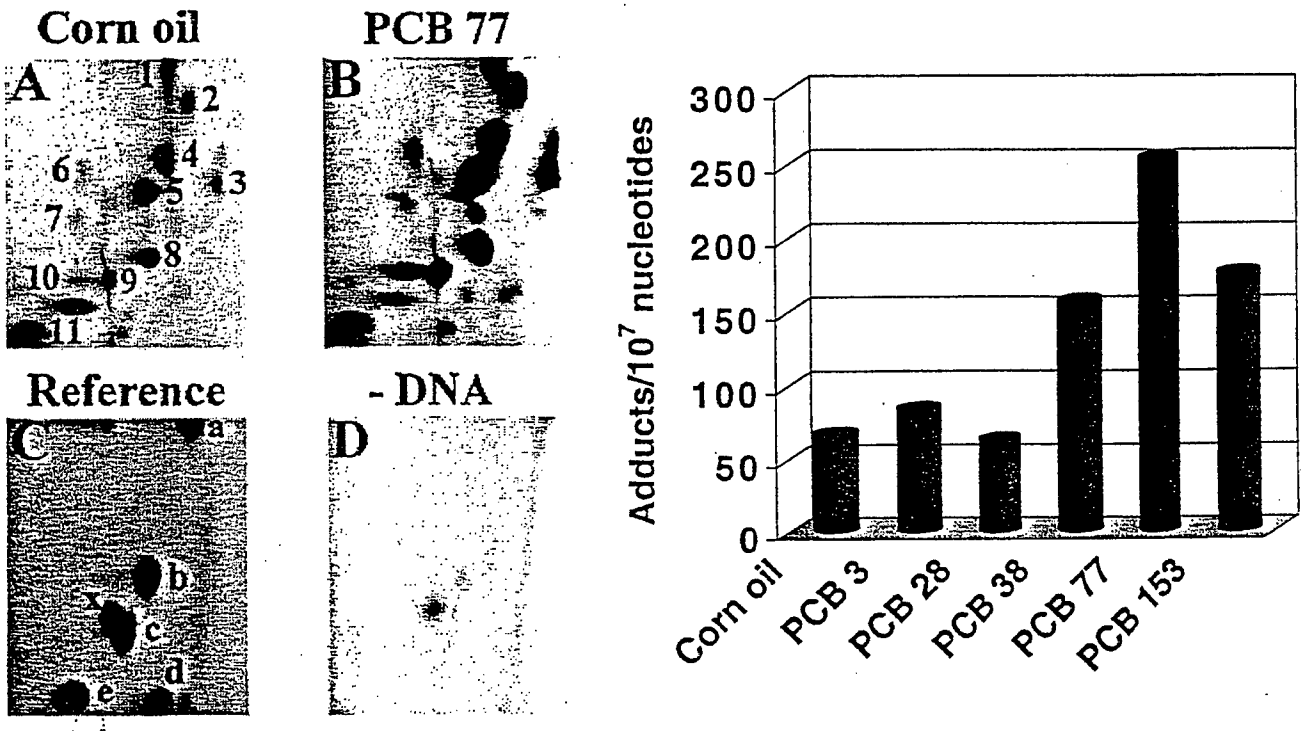


Figure: Effect of PCB treatment on the levels of endogenous polar DNA adducts in rat livers. LEFT: ^{32}P- adduct maps; A, vehicle treatment; B, treatment with PCB 77; C, reference adducts: a, etheno-dA, b, crotonaldehyde, c, hexenal, d, thymidine glycol, e, MDA + 4-HNE, x, radioactive contaminant; D, no DNA. RIGHT: adduct levels quantified.

Indirect evidence of oxidative stress: activation of transcription factors

As we reported earlier, we observed an activation of the transcription factor AP-1 in rats, treated with non-coplanar PCBs. Further analysis showed increased binding of the jun-B protein to AP-1 to the consensus sequence in livers of rats treated with 4-, 2,4,4'- or 2,2',4,4',5,5'-CBP. 3,4-CBP and 3,3',4,4'-CBP were inactive in this assay. AP-1 is known to be induced by intracellular oxidative stress. 2,4,4'- and 2,2',4,4',5,5'-CBP also induced increased binding of STAT to the GAS consensus element in these liver nuclei. The activation of the transcription factors AP-1 and STAT by these non-coplanar PCBs points toward oxidative stress as a result of PCB treatment. We therefore

dedicated considerable efforts to further analyze the production of ROS during PCB metabolism and the consequences *in vitro* and *in vivo*.

Production of reactive oxygen species (ROS) during PCB metabolism and DNA damage *in vitro*.

Since PCBs are metabolized by liver and breast microsomes to dihydroxy metabolites, and since a lot of findings point towards indirect mechanisms of PCB toxicity, it was of interest to learn more about the reactivity of this class of compounds. First studies were done in aqueous buffers *in vitro*. We already reported that dihydroxy-PCBs produce ROS *in vitro* by enzymatic or non-enzymatic oxidation to the quinone. One enzyme that was able to oxidize these compounds to the quinones was lacto-peroxidase. This breast specific enzyme was equally efficient as horseradish peroxidase or myeloperoxidase, suggesting that further metabolic activation of hydroxylated PCBs can occur in the breast. We showed also that quinones are not the endpoint, but that even more ROS are formed by redox cycling or reaction of the quinones or with glutathione (GSH). To summarize this: dihydroxy-PCBs can be oxidized by lactoperoxidase to quinones, which redox-cycle and bind to GSH, all resulting in the production of ROS.

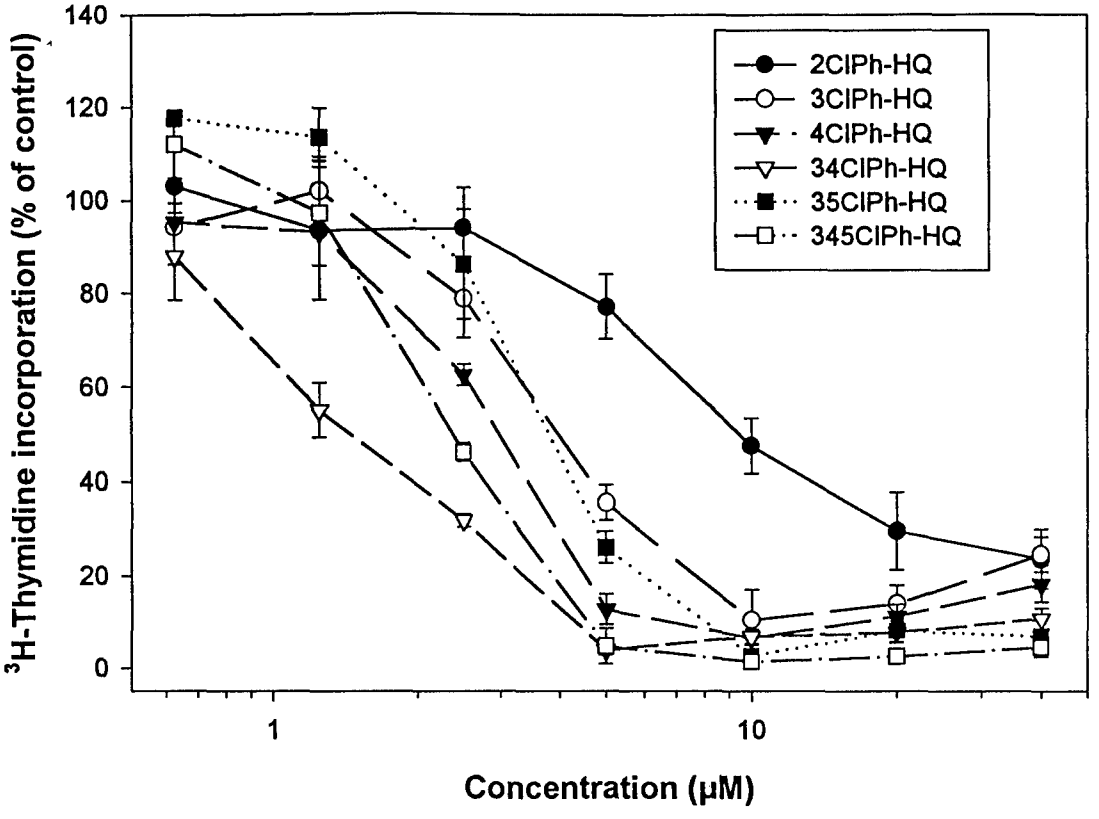
We wanted to know, of course, whether this PCB-generated ROS production could lead to DNA damage. Two typical kinds of DNA damage induced by ROS are DNA strand breaks and formation of 8-oxo-deoxyguanine (8-OH-dG). As we reported previously the ROS production by dihydroxy- and quinone PCB metabolites resulted in DNA strand breaks *in vitro* in the presence of Cu(II). We also found that the exposure of DNA to dihydroxy-PCBs *in vitro* in the presence of lactoperoxidase resulted in a significant increase in 8-OH-dG. Although these effects were *in vitro* they nevertheless indicate that similar reactions may happen *in vivo* and thereby could result in cancer initiation. The next step was to analyze the effect of these PCB metabolites (dihydroxy- and quinone-PCBs) in cells in culture.

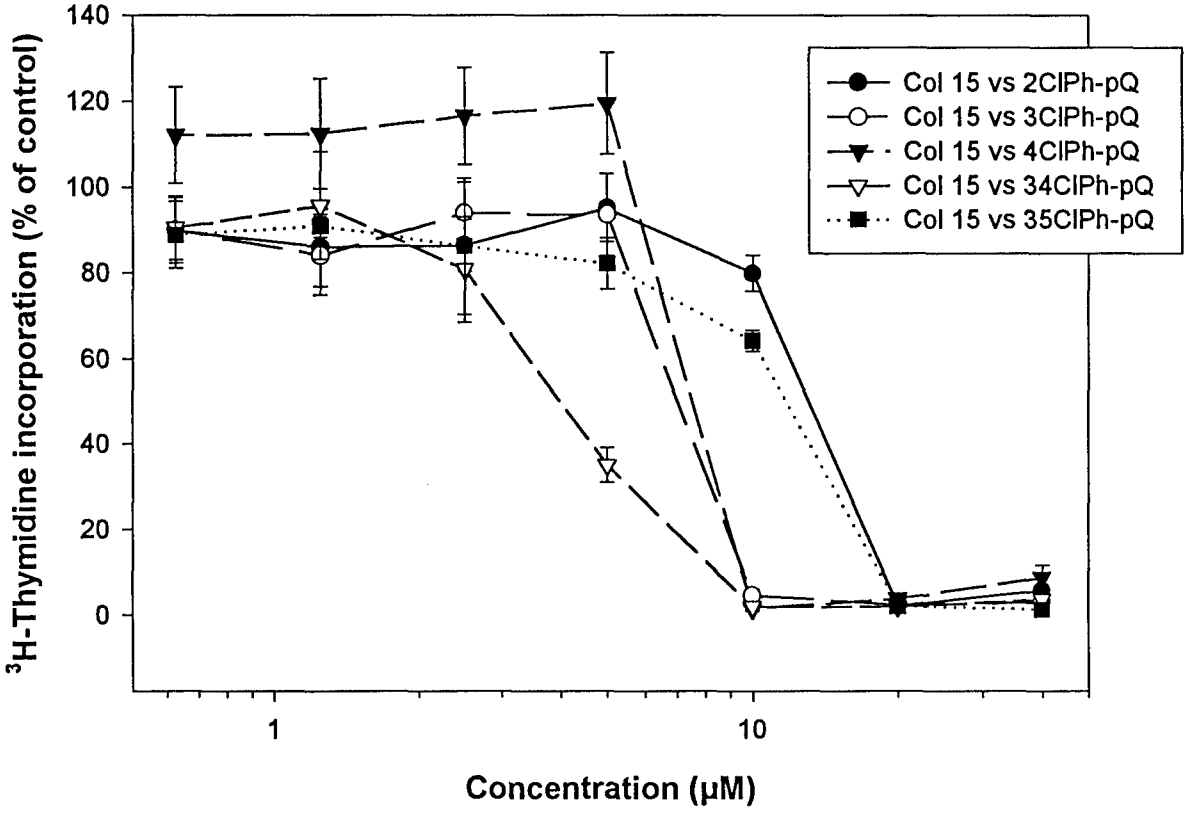
Effect of PCB metabolites on cells in culture: Toxicity, ROS production, and GSH depletion

In order to mimic breast tissue and to optimize our assays for these compounds, dihydroxy- and quinone-metabolites, we wanted to use a cell line that expresses a peroxidase. Since we could not identify a lactoperoxidase positive breast cell line, we decided to use a cell line that contains high myeloperoxidase activity instead. This choice seems justifiable, since we had shown before that both enzymes oxidize dihydroxy-PCBs to the same extent. The cell line chosen is the human leukemia line HL-60. Dihydroxy-metabolites with the hydroxy-groups in *para*-position, so called hydroquinones (HQ) and their quinones (Q) were used.

a. Cytotoxicity

We first analyzed the toxicity of these metabolites by measuring ^{3}H-thymidine incorporation after 1 day of exposure ($5x10^4$ cells/ml medium/5%FCS). Dihydroxy- and quinone PCB metabolites with 1 to 3 chlorines in different positions in the second ring were used. The data are the mean of triplicates expressed as percent ^{3}H-thymidine incorporation compared to solvent control (Figures on next page). The LD_{50} of all hydroquinone and quinone-metabolites tested was in the range of 1 to 10 µM. The


140
120
100
80
60
40
20
0
1
10
^{3}H-Thymidine incorporation (% of control)
Concentration (µM)
2ClPh-HQ
3ClPh-HQ
4ClPh-HQ
34ClPh-HQ
35ClPh-HQ
345ClPh-HQ


140
120
100
80
60
40
20
0
1
10
^{3}H-Thymidine incorporation (% of control)
Concentration (µM)
Col 15 vs 2ClPh-pQ
Col 15 vs 3ClPh-pQ
Col 15 vs 4ClPh-pQ
Col 15 vs 34ClPh-pQ
Col 15 vs 35ClPh-pQ

2-Cl metabolites were always the least toxic and the 3,4-diCl metabolites the most toxic. This may indicate a higher toxicity of *para-* vs *ortho*-chlorinated biphenyls, but the toxicity curves of all compounds were too similar to make a strong statement about structure-activity relationships.

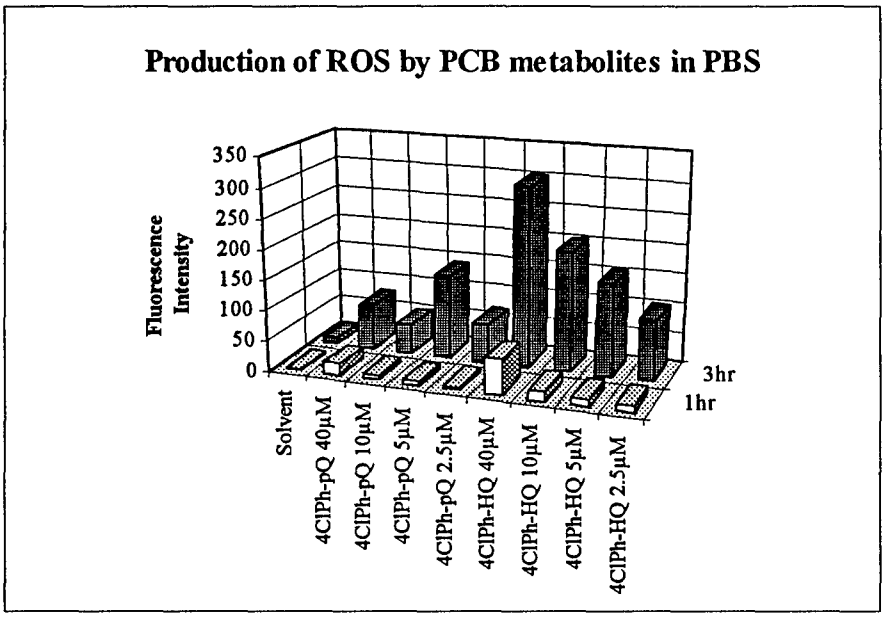
b. Intracellular ROS production

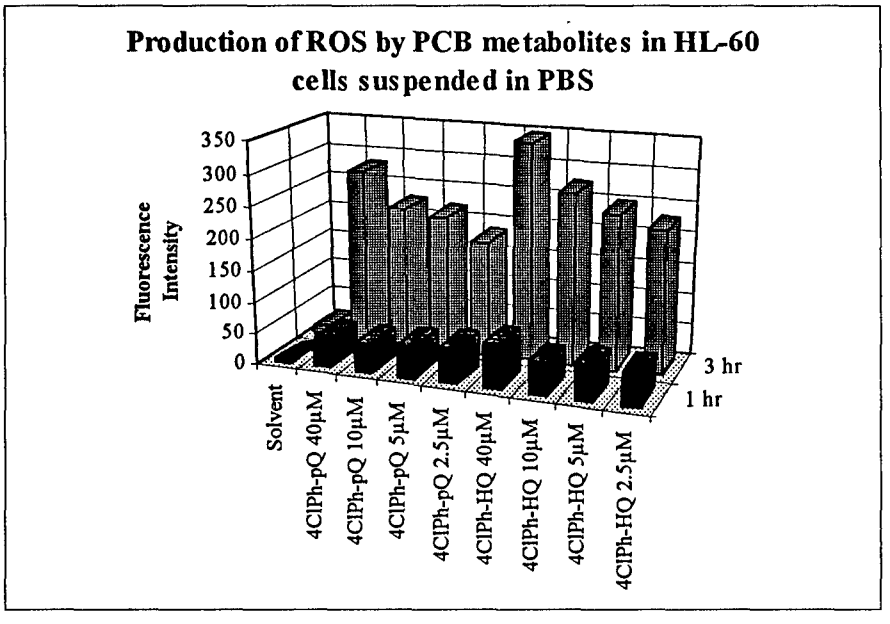
One possible mechanism of toxicity is the production of intracellular ROS. A common method to measure intracellular ROS production is with 2',7'dichlorodihydrofluorescein diacetate (DCF). This non-fluorescent dye can migrate into living cells. When exposed to peroxides it is changed to a fluorescent dye. The fluorescence can be measured in a fluorometer at 485 nm excitation and 530 nm emission wavelengths. We exposed HL-60 cells ($1x10^5$/ml PBS, 3 ml per sample) to different concentrations of the dihydroxy- and quinone metabolites of 4-CBP and measured fluorescence after 1 hr and 3 hrs of exposure. Since we know that dihydroxy-PCBs can autoxidize with the production of superoxide, which dismutates to hydrogen peroxide, we also measured fluorescence in PBS + compound without cells. The difference should give us the cell-mediated ROS production.

Table: Fluorescence Intensity (units) after incubation of 4-CBP metabolites in buffer or buffer with cells for 1 or 3 hrs at different concentrations.

Compound μM	**Buffer**	**Buffer + Cells**	**Cells alone (calculated)**	**Buffer**	**Buffer + Cells**	**Cells alone (calculated)**
	1 hr	*1 hr*	*1 hr*	*3 hrs*	*3 hrs*	*3 hrs*
Solvent	1.01 ± 0.35	4.16 ± 0.40	3.15	10.99 ± 5.93	17.05 ± 0.83	6.06
4ClPh-pQ						
2.5	5.55 ± 1.14	53.38 ± 3.07	47.82	64.58±10.79	184.72±11.5	120.13
5.0	5.77 ± 0.35	48.91 ± 1.12	43.13	141.39±67.4	217.43±26.9	76.04
10	8.43 ± 1.13	44.70 ± 1.40	36.26	49.35 ± 5.50	227.12±15.4	177.76
40	18.49 ± 0.31	47.42 ± 4.56	28.92	76.18±13.05	281.45±2.16	205.27
4ClPh2-HQ						
2.5	10.91 ± 4.91	51.93 ± 0.40	41.01	100.97±13.3	226.97±7.52	125.19
5.0	10.58 ± 0.30	51.85 ± 4.70	41.27	155.03±10.9	246.65±17.3	91.62
10	16.62 ± 1.07	48.94 ± 1.92	32.31	199.56±10.2	272.65±7.23	73.09
40	57.62 ± 3.99	68.13 ± 2.17	10.50	300.01±23.0	345.86±4.32	45.84

The Table above shows the mean fluorescence plus/minus SD of triplicate samples. Cells alone in phosphate buffered saline solution (PBS) produced only a small increase in fluorescence. The fluorescence increased with time. A small increase in fluorescence was even seen when no cells or compound was present. However, when PCB metabolites were added to PBS, a sharp increase in fluorescence was seen. Compound in buffer, without cells, resulted in a time and dose dependent increase in fluorescence intensity that was higher with the hydroquinones then with the quinones (Figure below). This is most likely due to autoxidation of the hydroquinone and non-enzymatic redox reactions of the quinone. In the presence of HL-60 cells, all hydroquinone and quinone metabolites tested resulted in a significant increase in fluorescence above buffer-controls, indicating intracellular peroxide formation. After





1 hr incubation no dose-response could be observed. After 3 hrs incubation the increase in fluorescence was nearly 5x higher than after 1 hr and dose-dependent. Again the hydroquinone was more active than the quinone. A surprising effect was seen when the two samples, with and without cells, were subtracted from each other to calculate the fluorescence due to cellular-mediated processes (see Table). Here the dose-response seems to be reversed for the hydroquinone and 1 hr quinone samples. At this time we can only speculate about the mechanism. Since the treatments resulted in severe toxicity, it is possible that this cytotoxicity reduced the cell-mediated effect with increasing concentrations. The quinones, however, are activated by conjugation with GSH. Increased toxicity may facilitate the access of the quinone to GSH and thereby increase

ROS production. More experiments with lower concentrations are planned to elucidate this paradox.

c. Depletion of GSH and inhibition of Topoisomerase II

All *in vitro* and *in vivo* data suggested that the reaction of the quinone-metabolites with GSH may play a role in toxicity and possibly indirectly in genotoxicity and carcinogenicity, by depletion of the major protective peptide against ROS in exposed cells. We therefore quantified the binding of PCB hydroquinones and quinones to GSH *in vitro* and examined depletion of intracellular GSH in HL-60 cells *in vivo.*

The binding of GSH to the PCB metabolites *in vitro* was determined by incubating 100 μM compound with 50, 100, 200, and 400 μM GSH in PBS for 1 hr, after which the remaining amount of free GSH was determined by 2 methods: measurement of fluorescence due to GSH-bound monobromobimane (MBB) and spectrometric measurement of TNB formation from DTNB by GSH oxidation in an GSSG-GSH enzyme cycling method (Tietze et al., 1969). From the resulting data the moles of GSH bound per mole test compound was calculated. The MBB assay is fast and inexpensive, but less specific for GSH and measures only reduced GSH. The enzyme cycling method is specific for the peptide and measures total GSH (GSH + GSSG). The Table below shows that after 1 hr incubation *in vitro* hardly any GSH was bound to the hydroquinone metabolites. The quinones bound GSH at a ratio of about 1 : 1.5.

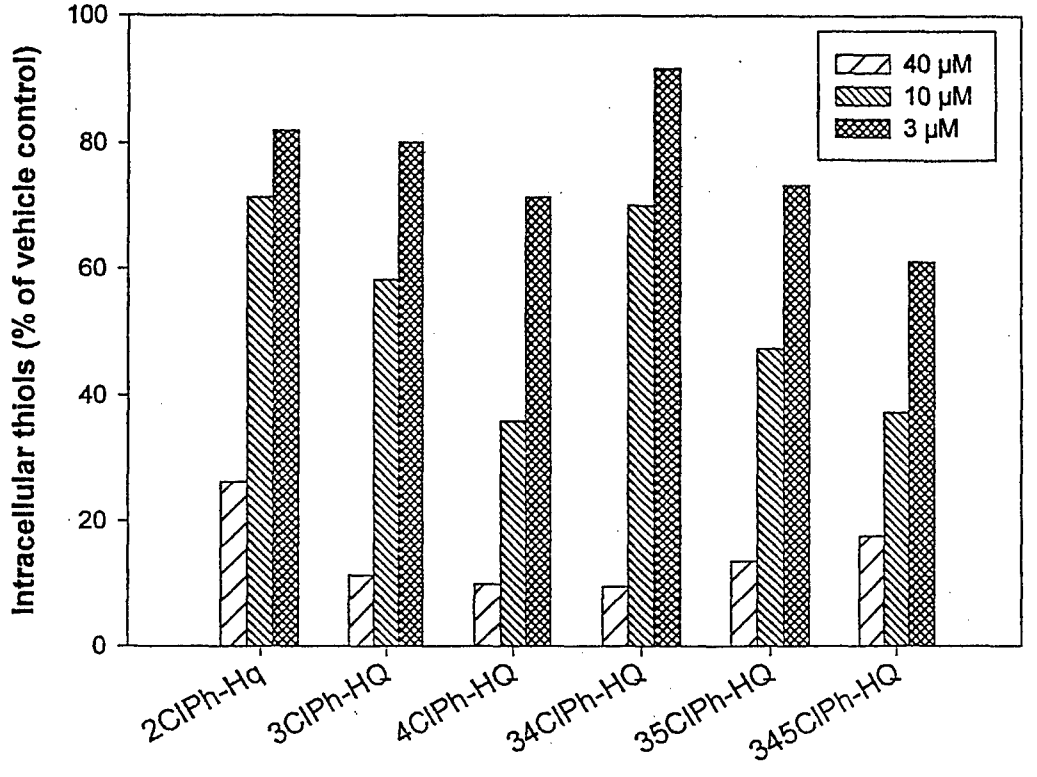
Table: Moles of GSH bound per mole PCB-metabolite

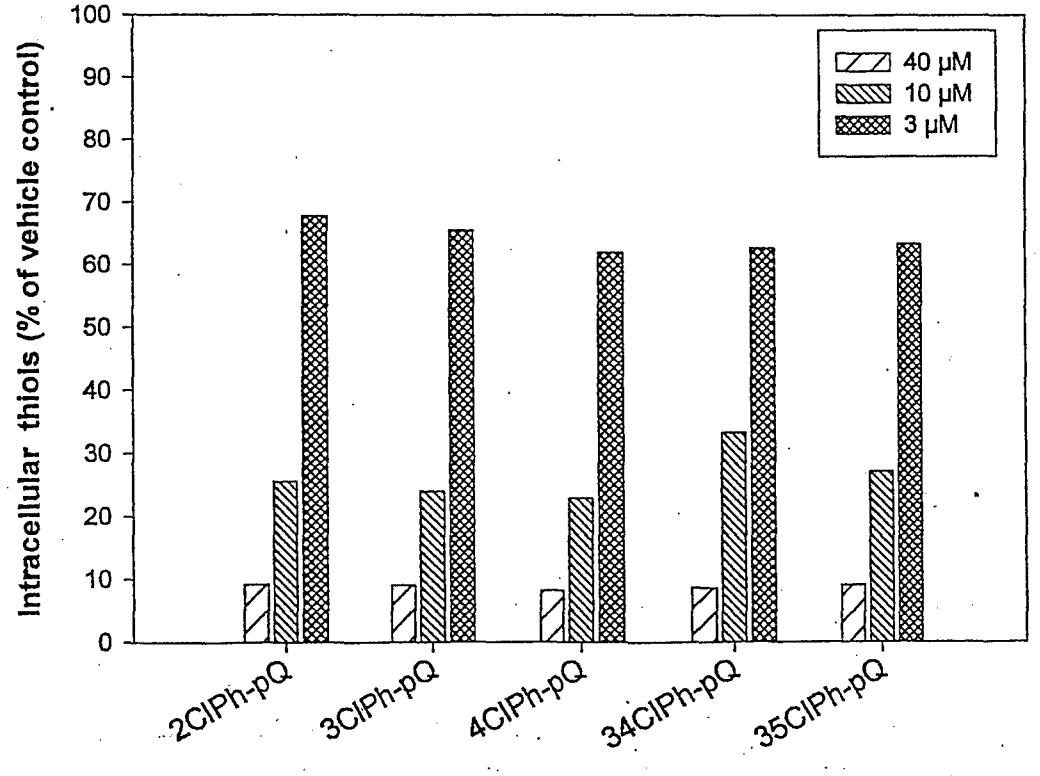
PCB-Quinone	GSH bound (enzyme meth.)	GSH bound (MBB method)	PCB-Hydroquinone	GSH bound (enzyme meth.)	GSH bound (MBB method)
2ClPh-pQ	1.29	1.39	2ClPh-HQ	0.15	n.d.
3ClPh-pQ	1.33	1.36	3ClPh-HQ	0.22	n.d.
4ClPh-pQ	1.43	1.64	4ClPh-HQ	0.15	n.d.
3,4ClPh-pQ	1.35	1.32	3,4ClPh-HQ	0.46	n.d.
3,5ClPh-pQ	1.94	1.64	3,5ClPh-HQ	0.31	n.d.
3,4,5ClPh-pQ	n.t.	n.t.	3,4,5ClPh-HQ	0.51	n.d.

n.t. not tested n.d. none detectable

To measure intracellular GSH depletion $1x10^6$ cells/ml PBS (3 ml per sample) were incubated with different concentrations of test compound for the indicated amount of time and then the remaining GSH determined with MBB and with the enzyme cycling method. The following Figures show the results of an MBB assay with several PCB hydroquinones and quinones after 1 hr incubation with 3, 10, or 40 μM compound. Hydroquinones at 3 μM concentration reduced intracellular reduced GSH by about 80%, 10 μM to about 50%, and 40 μM to about 20% of control. The 4-Cl metabolite was more active then the 2-Cl metabolite. An increase in chlorination did not result in significantly different GSH depletion. There was no structure-activity relationship with the PCB-quinones. The remaining intracellular GSH was about 60% of control at 3 μM, 25% at 10 μM, and 9% at 40μM. Obviously the effect of the quinones was stronger, probably because they could react directly with GSH. However, the significant reduction of intracellular GSH by the hydroquinones indicates that these metabolites are also active, probably by enzymatic (peroxidase!) or non-enzymatic oxidation to the quinone. This means they could cause a reduction of intracellular GSH by two mechanisms: oxidation

to GSSG by ROS that are produced during hydroquinone oxidation and by conjugation to the resulting quinone. If this were true, we should see less GSH depletion with the enzyme cycling method.





The Table below shows the percent of remaining intracellular GSH compared to control after incubation of cells for 1 hr with 2-CBP metabolites. GSH was determined by both methods, enzyme cycling and MBB. The amount of total GSH in control cells, measured by the enzyme cycling method, was about 2.5 nmoles/10^6 cells. This is in agreement with the literature. Incubation of HL-60 cells for 1 hr with 5 to 40 μM of the 2ClPh-hydroquinone resulted in only small, non-significant reductions in total GSH. The same amounts of the corresponding quinone reduced the intracellular total GSH (enzymatic method) or reduced GSH (MBB method) in a dose-dependent fashion. Depletion of reduced GSH at 5 and 10 μM concentrations seems to be stronger than depletion of total GSH. This could be due to differences in the accuracy of the 2 assays or in an increased GSH oxidation during treatment with PCB metabolites. We are in the process of measuring intracellular GSSG in these samples to clarify these possibilities.

Table: Remaining intracellular GSH in % of control cells after 1 hr incubation with 2-CBP metabolites.

Concentration (μM)	2ChPh-HQ (Enzymatic Method)	2ClPh-pQ (Enzymatic Method)	2ChPh-pQ (MBB Method)
1.25	n.t.	n.t.	85 %
5	88 %	86 %	55 %
10	92 %	71 %	21 %
40	82 %	2 %	10 %

We were interested to see how longer exposure times would influence the effect of 2-CBP metabolites on intracellular GSH. The following Table shows preliminary data (mean of triplicates) from 1 experiment (enzyme cycling method), where cells were incubated with 5 μM of the hydroquinone or quinone for up to 6 hrs. The hydroquinone initially caused a small reduction of intracellular GSH. After 3 and 6 hrs, however, intracellular GSH seemed to be increased compared to controls. This could be due to a feedback mechanism in the cells, resulting in increased GSH production after smaller insults. None of these changes were statistically significant, however. With the quinone a significant reduction of intracellular GSH was observed after 6 hrs of exposure (p value of 0.001; Fishers LSD test). More experiments are needed to confirm these data.

Table: Intracellular GSH (% of controls) after exposure for 1 – 6 hrs to 2-CBP metabolites.

Exposure Time	2ClPh-HQ	2ClPh-pQ
1 hr	87%	84%
3 hrs	108%	86%
6 hrs	116%	50%

Since the PCB metabolites have such a strong effect on intracellular GSH, it seems possible that part of the toxicity could be due to binding to other sulfhydryl containing cellular macromolecules. One important nuclear molecule is topoisomerase II (Topo II). This enzyme is essential for unwinding of the double helix for DNA synthesis, repair, and other processes. Inhibition of this enzyme could have deleterious consequences for the cells. Topo II activity can be measured by incubating high molecular weight kinetoplast DNA (kDNA) with Topo II with/without test compound, followed by gel electrophoreses. Normal activity of Topo II results in decatenation of the

kDNA and migration into the gel. If Topo II is inhibited, the kDNA stays in the well. We tested the activity of Topo II after a 1 hr incubation with 2ChPh-HQ and 2ClPh-Q *in vitro*. Up to 100 μM of the hydroquinone had no effect on the activity of Topo II (data not shown). The quinone inhibited Topo II activity in a dose-dependent way (see Figure below). This inhibition of Topo II activity by 100 μM 2-ClPh-pQ was significantly reduced when 50 μM GSH was added to the incubation mixture and completely inhibited 0 μM (solvent control, 11); kDNA + 100 μM compound alone, by 100 μM GSH or more (data

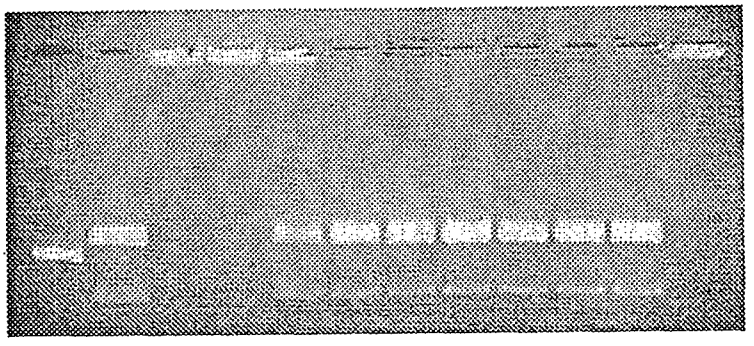


Figure: Inhibition of topoisomerase II activity by 2ClPh-pQ. Line 1, linear plasmid control; 2, decatenated kDNA control; 3, kinetoplast DNA without Topo II; 4-10, kDNA, Topo II, and decreasing amounts of 2ClPh-pQ: 100 μM (4), 5 μM (5), 25 μM (6), 10 μM (7), 5 μM (8), 2.5 μM (9), 1 μM (10), 0 μM (solvent control, (11); line 12, kDNA + 100 μM compound alone.

not shown).

These results show that PCB quinones and to a lesser degree PCB hydroquinones strongly react with cellular sulfhydryl containing proteins like Topo II and peptides like GSH. This may result in cytotoxicity or lead to compromised cells, where essential molecules are not working in the optimal way and where cellular organelles are exposed to oxidative stress, because the protection by intracellular GSH is reduced. Both mechanisms could be involved in PCB toxicity and carcinogenicity, if they occur in the live animal *in vivo*.

Production of 8-OH-dG in rats *in vivo* by treatment with PCBs

Previous results

We previously reported that in first experiments using the ^{32}P-postlabeling method a 2-fold increase of 8-OH-dG was seen in the livers of rats treated *in vivo* with 3,4-CBP (2 samples only). To our surprise, the level of 8-OH-dG was not changed or even decreased after treatment with 4-, 2,4,4'-, 3,3',4,4'- or 2,2',4,4',5,5'-CBP.

When female Sprague/Dawley rats were treated with 3,4-CBP or 3,4,5-CBP as described above, the amount of 8-OH-dG/10^6 nucleotides in mammary tissue was even lower than in the vehicle-treated control. When mammary tissue from animals that had been pre-treated with PB + β-NF or higher chlorinated PCBs for enzyme induction, as described above, were analyzed using the same ^{32}P-postlabeling assay conditions as used for the *in vitro* experiment, no significant increase in 8-OH-dG over vehicle treatment

was detected. One problem in these studies was, however, the relatively high background of 8-OH-dG, which possibly could obscure small, but exposure-related increases.

Improved method and new *in vivo* studies

Recently several reports, including our own work (Gupta & Arif, 1998), have shown that artifactual production of 8-OH-dG may interfere in detecting the induction of this oxidative lesion *in vivo*, unless special measures are taken. In our last report, we described several possible factors responsible for the artifactual production of 8-OH-dG during the isolation of DNA. To solve this problem we tested several known antioxidants to trap the free radicals generated during the isolation. 8-Hydroxyquinoline (8-HQ) and N-t-butyl-α-phenylnitrone (PBN) were found to be most effective against the artifactual production of 8-OH-dG.

Using these improved conditions no significant increase in 8-OH-dG was observed compared to the vehicle controls (4.7 ± 1.0 8-OH-dG/10^6 nucleotides), when the livers of animals that were treated only with 3,4-CBP or 3,3',4,4'-CBP were analyzed by TLC enrichment-mediated ^{32}P-postlabeling technique. However, in combination with pre-treatment for enzyme induction by PB+β-NF, 3,3',4,4'-CBP treated animals had significantly higher levels of 8-OH-dG (7.5 ± 0.5 8-OH-dG/10^6 nucleotides) in liver DNA; 3,4-CBP was found ineffective.

In our continuing efforts to minimize the artifactual production of 8-OH-dG, we later found out that artifactual 8-OH-dG could still be produced during processing of DNA. In an attempt to learn more about the mechanisms of artifactual 8-OH-dG production, we carried out a series of experiments under various conditions, among them: *i*) with or without fluorescent light, and *ii*) with or without use of protectants during DNA processing. Briefly, when the rat liver DNA was processed in the absence of direct fluorescent light, artifactual 8-OH-dG production was substantially suppressed compared with the normal fluorescent light working conditions. Further, when the DNA was digested in the presence of 8-HQ/PBN under no direct fluorescent light, 8-OH-dG measurements by ^{32}P-postlabeling-TLC showed additional 50% reduction in the artifactual production of 8-OH-dG. The 8-OH-dG values (0.95 ± 0.12 8-OH-dG/10^6 nucleotides) obtained in rat liver DNA by the modified procedure were in the vicinity of 8-OH-dG values reported by HPLC-ECD. We already have breast epithelium and livers from rats treated with several PCBs as described in the previous report. The DNA of this mammary tissue will be isolated and 8-OH-dG analysis will be performed under the modified conditions.

The fact that enzyme induction was needed points to the possibility that under normal conditions the balance of metabolic activation and detoxification is such that rat mammary tissue may be protected from damage by acute exposure to these PCBs. In real life, of course, be have to expect exposure to low levels of compounds over long periods of time, in fact our whole lifetime, and not only to PCBs but also to other xenobiotics. Moreover, several of these compounds, including PCBs, are known to increase or decrease enzyme levels. This could significantly change the balance between activation and detoxification. This rose the question, whether exposure to relatively low chlorinated PCBs itself could modulate the defense system in exposed animals in a way that would make them more vulnerable to PCBs or other toxic compounds.

Antioxidant defense mechanisms in the rat liver after PCB treatment and in human breast tissue

The level of oxidative stress in cells is strongly influenced not only by exposure to ROS producing compounds, but also indirectly by changes in the level of protective enzymes or cofactors or xenobiotic activating or ROS producing enzymes. As previously reported we analyzed several of these indirect modulators of oxidative stress and how they are influenced by PCBs. We found that treatment with medium to high chlorinated PCBs (3,3',4,4'-CBP and 2,2',4,4',5,5'-CBP) increased cytochromes P450, glutathione transferase (GST) and GSSG reductase activity and decreased catalase activity in livers of female rats. We found that treatment with either of both of these PCBs resulted in increased lipid peroxidation in the liver 6 days after treatment. This is in agreement with our finding (above) of increased polar endogenous DNA adducts, which we believe are the result of lipid peroxides. In addition, when animals who had been treated with these higher chlorinated PCBs were then challenged with lower chlorinated PCBs (3,4- or 3,4,5-CBP) a significant decrease in the amount of GSH was observed. This decrease in protective GSH should make the cells more vulnerable to even low levels of exposure to ROS or electrophiles.

Rat breast tissue is very small, and we therefore postponed similar analysis in this tissue. An analysis of the level of antioxidant enzymes in human breast tissue seemed by far more informative. This assumption was correct. As reported previously that we observed enormous inter-individual differences in the levels of the 3 enzymes tested in human mammary tissue, Se-GSH peroxidase (Se-GPx), GSH transferase (GST), and catalase. Among 6 individuals the activity varied from 1.8 – 30 U/mg protein for Se-GPx, from 22 – 112 mU/mg protein for GST, and from 1.7 – 124 U/mg protein for catalase. This shows that the risk from PCB exposure may vary strongly from woman to woman.

All these results assure us that it is of utmost importance to understand the mechanisms of PCB carcinogenicity. This knowledge is an essential prerequisite for defining conditions that could lead to cancer induction by PCBs. Knowing how everything interacts is necessary to identifying women who are at risk for cancer development by PCB exposure due to their individual enzyme composition (activity and polymorphism) and to possibly finding ways to protect these women by modulating their diet and lifestyle.

SUMMARY AND CONCLUSIONS

We want to learn more about the mechanisms of PCB carcinogenesis and about the possibility that these mechanisms could occur in human breast tissue, where PCBs accumulate. So far we have gained the following insights:

1. We could prove for the first time that a PCB, the 4-CBP, can act as an **initiator** in the Solt-Farber rat liver carcinogenesis model.

2. We could also show that the tested PCBs, who are fairly inactive themselves, bind covalently to nuclear protein and DNA in the livers of mice. This means that they were metabolically activated to electrophiles. The strong binding to protein suggests that quinones may have been formed, since these metabolites have a high reactivity with cellular sulfhydryl groups. Preliminary data (not included in this reported) that PCBs are metabolized to compounds that bind to sulfydryl groups in hemoglobin further strengthen our hypothesis that PCB-quinones may plays an important role *in vivo*.

3. Since we had already previously reported that the metabolism of PCBs by rat liver and human breast microsomes differs only quantitative, not qualitative, we believe that metabolic activation of PCBs to an initiating carcinogen that occurs in the rat liver may also occur in the human breast.

4. The first possible mechanism of cancer initiation that we analyzed was DNA adduct formation by PCB metabolites. We could show that PCB-quinones can react *in vitro* with DNA to form adducts. The migration pattern of these adducts of synthesized quinones with DNA can now be used as standards. However, 4-chlorophenyl-hydroquinone was about 4-8 times more active in DNA adduct formation then the 4-chlorophenyl-quinone. This could mean that a semiquinone or other hydroquinone derivative is the major adduct forming species.

5. We had problems to show DNA adduct formation by PCBs *in vivo* even in the most promising target organ, the liver. We plan next to use a cell culture model to search for PCB-DNA adducts. The results of these cell culture methods will be used as an indicator for possible mechanisms *in vivo*.

6. We found repeatedly evidence that ROS are formed *in vivo* during PCB exposure.
   a. The activation of the transcription factors AP-1 and STAT by these non-coplanar PCBs points toward oxidative stress as a result of PCB treatment.
   b. In combination with pre-treatment for enzyme induction by PB+β-NF, 3,3',4,4'-CBP treated animals had significantly higher levels of 8-OH-dG (7.5 ± 0.5 8-OH-dG/10^6 nucleotides) in liver DNA; 3,4-CBP was found ineffective.
   c. We found that treatment with medium to high chlorinated PCBs, PCB 77 and/or PCB 158, resulted in increased lipid peroxidation in the liver of rats 6 days after treatment.

d. Three of five PCBs (PCB 38, PCB 77 and PCB 153) produced a significant increase in the levels of endogenous polar DNA adducts in rat livers. Such polar adducts are formed by ROS through lipid peroxidation.

7. ROS formation may be a result of redox-reactions by dihydroxy-PCBs/quinones. We could prove that dihydroxy-PCBs can be oxidized *in vitro* by peroxidases, including lactoperoxidase, to quinones, which redox-cycle and bind to GSH, all resulting in the production of ROS. The presence of lactoperoxidase may make the breast specifically vulnerable. We also found that in the presence of myeloperoxidase containing HL-60 cells, all PCB hydroquinone and quinone metabolites tested resulted in a significant increase in fluorescence above buffer-controls, indicating intracellular peroxide formation.

8. Oxidative stress, however, could also result from changes in pro-oxidant/anti-oxidant enzymes and cofactors. We observed that
   a. PCB quinones and to a lesser degree PCB hydroquinones strongly react with intracellular sulfhydryl containing proteins like Topo II and peptides like GSH. GSH depletion would make the cells more vulnerable to damage by ROS.
   b. Treatment with medium to high chlorinated PCBs (PCB 77 and PCB 158) increased cytochromes P450 (pro-oxidant), glutathione transferase (GST) and GSSG reductase activity and decreased catalase activity in livers of female rats.

9. We observed enormous inter-individual differences in the levels of the 3 enzymes tested in human mammary tissue, Se-GSH peroxidase (Se-GPx), GSH transferase (GST), and catalase. This may mean that individual women may be at higher risk due to their individual enzyme activities.

These results confirm our hypothesis that PCBs can be metabolized to compounds that directly (DNA adduction) or indirectly (ROS production and/or changing of the anti-oxidant status of the cell/tissue). Experiments are under way to further elucidate the mechanism of PCB carcinogenesis. The strong findings about ROS let us to believe that they may be the key and causes us to broaden our research efforts to include the polar endogenous adducts in our future analysis of breast tissue. The large inter-individual differences in metabolic abilities in humans assure us that it is very important to understand the mechanisms, since only this knowledge can lead to protection and intervention.

KEY RESEARCH ACCOMPLISHMENTS

1. We could prove for the first time that a PCB, the 4-CBP, can act as an **initiator** in the Solt-Farber rat liver carcinogenesis model.

2. We could also show that the tested PCBs, who are fairly inactive themselves, bind covalently to nuclear protein and DNA in the livers of mice.

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4. The first possible mechanism of cancer initiation that we analyzed was DNA adduct formation by PCB metabolites. We have shown that PCB-quinones can react *in vitro* with DNA to form adducts. .

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REPORTABLE OUTCOMES

Abstracts and Presentations in 1998 and 1999

137. B. Hennig, R. Slim, M. Toborek, A. Daugherty and L.W. Robertson. PCB-mediated endothelial cell dysfunction: Implications in atherosclerosis. Presented at the 19th Symposium on Halogenated Environmental Organic Pollutants and POPs (DIOXIN '99) (Venice, September 12-17, 1999). Organohalogen Compounds 41, 45-49, 1999.

136. H. P. Glauert, J.C. Tharappel, E.Y. Lee, L.W. Robertson and B.T. Spear. Activation of the transcription factors NF-κB and AP-1 during the promotion of hepatocarcinogenesis by Polychlorinated Biphenyls (PCBs). Presented at the 19th Symposium on Halogenated Environmental Organic Pollutants and POPs (DIOXIN '99) (Venice, September 12-17, 1999). Organohalogen Compounds 42, 413-416, 1999.

135. G. Ludewig, H.-J. Lehmler, Å. Bergman and L.W. Robertson. Metabolites of biphenyls and halogenated biphenyls: reactivity of dihydroxylated biphenyls *in vitro* and *in vivo*. Presented at the 19th Symposium on Halogenated Environmental Organic Pollutants and POPs (DIOXIN '99) (Venice, September 12-17, 1999). Organohalogen Compounds 42, 455-458, 1999.

134. H.-J. Lehmler, L.W. Robertson, Å. Bergman, C. P. Brock and B. Patrick. Synthesis of Polychlorinated Biphenyls (PCB) and Metabolites: Tools to Investigate Their Metabolic Activation. Presented at the Semi-Annual Meeting of the American Chemical Society in New Orleans, LA, August, 22-26, 1999.

133. H.-J. Lehmler, G. Ludewig, Å. Bergman, L.W. Robertson. Oxidation of Halogenated 4,4'-Biphenols by Horseradish Peroxidase. Presented at the 31st Central Regional Meeting of the American Chemical Society in Columbus, Ohio, June 21-23, 1999.

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131. J.C. Tharappel, L.W. Robertson, E.Y. Lee, B.T. Spear and H.P. Glauert. Effect of the PCBs 2,2',4,4',5,5'-hexachlorobiphenyl (PCB-153) and 3,3',4,4'-tetrachlorobiphenyl (PCB-77) on cell proliferation and the activation of the transcription factors NF-kB and AP-1 in rats treated with diethylnitrosamine (DEN). Presented at Experimental Biology 99, April 17-20, 1999 (Washington DC). Published in FASEB Journal, 13, Abstract Number 162.3.

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128. P. Espandiari, L.W. Robertson and G. Ludewig. Dihydroxybiphenyls: A structure-activity comparison. Presented at the 90th Annual Meeting of the American Association for Cancer Research, April 10-14, 1999 (Philadelphia, PA). Abstract Number 328.

127. T.P. Twaroski, J.M. Arif, R.C. Gupta, and L.W. Robertson. Antioxidant enzyme levels and oxidative damage in rats exposed to selected Polychlorinated Biphenyls (PCBs) after maximal cytochrome P450 induction. Presented at the 90th Annual Meeting of the American Association for Cancer Research, April 10-14, 1999 (Philadelphia, PA). Abstract Number 4269.

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120. J. de Boer, L.W. Robertson, F. Dettmer, H. Wichmann and M. Bahadir. Polybrominated diphenyl- ethers in human adipose tissue and relation with watching television – a case study. Presented at the 18th Symposium on Halogenated Environmental Organic Pollutants (DIOXIN '98) (Stockholm, 1998). Organohalogen Compounds 35, 407-410, 1998.

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120. P. Espandiari, H.P. Glauert, E.Y. Lee and L.W. Robertson. Promoting activity of the herbicide dicamba (2-methoxy-3,6-dichlorobenzoic acid) in two-stage hepatocarcinogenesis. International Journal of Oncology 14, 79-84 (1999).

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Students in training in 1998 and 1999

Tim Twaroski	Ph.D. Student 1996-2000	Issues of Oxidative Stress, Redox Status and Antioxidant Enzymes: Important Mechanisms in PCB Toxicity
Anandi Srinivasan	Ph.D. Student 1997-	Genotoxicity of PCBs and PCB metabolites: Structure-Function Relationships.
Nilufer Tampal	Ph.D. Student 1998-	Metabolism of PCBs: Transport, distribution and reactivity of PCB metabolites.
Daria Pereg	Ph.D. Student 1999-	Binding of PCBs to nuclear protein and DNA. (Joint with the University of Laval, Quebec, Canada)
Matthias Festag	Diplom Student 1999-2000	Cyto- and genotoxicity of PCBs and metabolites in blood, liver and intestine. (Joint with the University of Potsdam, Germany)

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